Published in final edited form as:

Dalton Trans. 2015 December 28; 44(48): 20972–20982. doi:10.1039/c5dt02837j.

Catalytic Metallodrugs Based on the LaR2C Peptide Target HCV SLIV IRES RNA

Martin James Ross¹, Seth S. Bradford¹, and J. A. Cowan^{1,2,*}

¹Evans Laboratory of Chemistry, The Ohio State University, 100 West 18th Avenue, Columbus, Ohio 43210

²MetalloPharm, 1790 Riverstone Dr., Delaware, OH 43015

Abstract

Prior work has demonstrated the potential effectiveness of a new class of metallopeptides as catalytic metallodrugs that target HCV IRES SLIIB RNA (Cu-GGHYrFK, **1**). Herein new catalytic metallodrugs (GGHKYKETDLLILFKDDYFAKKNEERK, **2**; and GGHKYKETDL, **3**) are described based on the LaR2C peptide that has been shown to bind to the SLIV HCV IRES domain. *In vitro* fluorescence assays yielded K_D values $\sim 10 \mu\text{M}$ for both peptides and reaction of the copper derivatives with SLIV RNA demonstrated initial rates comparable across different assays as well as displaying pseudo-Michaelis-Menten behavior. The sites of reaction and cleavage mechanisms were determined by MALDI-TOF mass spectrometry. The primary site of copper-promoted SLIV cleavage is shown to occur in the vicinity of the 5'-G₁₇C₁₈A₁₉C₂₀-3' sequence that corresponds to a known binding site of the RM2 motif of the human La protein and has previously been reported to be important for viral translation. This domain also flanks the internal start codon (AUG). Both copper complexes also showed efficacy in an HCV replicon assay ($IC_{50} = 0.75 \mu\text{M}$ for **2**-Cu, and $2.17 \mu\text{M}$ for **3**-Cu) and show potential for treatment of hepatitis C, complementing other marketed drugs by acting on a distinct therapeutic target by a novel mechanism of action.

Keywords

HCV; IRES; ATCUN motif; RNA cleavage; copper; La protein; LaR2C; SLIV; RRM

INTRODUCTION

Treatment of hepatitis C virus (HCV) infections is limited by the lack of an effective vaccine and toxicity as well as the development of resistance. To circumvent resistance problems, HCV therapy is expected to evolve towards multidrug therapy consisting of drugs with complementary mechanisms of action.¹ The catalytic metallodrugs described herein act on a

*Correspondence to: Dr. J. A. Cowan, Evans Laboratory of Chemistry, The Ohio State University, 100 West 18th Avenue, Columbus, Ohio 43210. Tel: 614 292 2703; Fax: 614 292 1685; cowan@chemistry.ohio-state.edu.

Supplementary Information.

MALDI-TOF peak assignments for each time point for reactions of **2**-Cu and **3**-Cu with SLIV in the presence of co-reagents, first order exponential fits, initial fits of distinct reaction products, gels, and illustrative figures are available as supplementary information.

target distinct from current HCV designed drugs (protease inhibitors, polymerase inhibitors, and the general antivirals interferon and ribavirin), as well as functioning by a novel mechanism, and so they have the potential to complement other therapeutic treatments under development.

Previous reports of catalytic metallodrugs has demonstrated the potential of this drug design strategy.²⁻⁹ The HCV internal ribosomal entry site (IRES) is a structured RNA present at the beginning of the viral mRNA and is important for the translation of viral proteins.¹⁰ The IRES initiates translation by recruiting proteins to form a complex that binds to the ribosome. Translation results in the synthesis of a single polyprotein precursor that is processed by the host and its viral proteases to form mature viral proteins for virus assembly. Structurally, the HCV IRES consists of 4 domains with distinct functions that lead to the initiation of translation (Figure 1 with stem-loop 1 omitted). HCV IRES RNA has been previously demonstrated to be important for the life cycle of the hepatitis C virus. The metallopeptide Cu-GGHYrFK-amide (**1**-Cu, Figure 2), consisting of a YrFK-amide motif for RNA binding and a Cu-GGH ATCUN (amino terminal copper and nickel-binding) motif to perform chemistry on the target, was shown to have high activity, both *in vitro* against HCV IRES stem loop IIb (SLIIb), and in an HCV cellular replicon assay.² In fact, it showed additive to slightly synergic activity when given in combination with recombinant interferon α -2b (rIFN α -2b). Herein is described a similar approach that has been applied to develop a new set of peptides targeting stem loop IV (SLIV) of the HCV IRES, which contains the AUG start codon. Prior studies have demonstrated the importance of SLIV and its potential as a target for therapeutic intervention, including targeting the GCAC sequences near the AUG start codon of the short hairpin RNA motif,¹¹ and the use of the LaR2C peptide to block binding of the full length protein.¹² Catalytic metallodrugs targeting SLIV should help to improve selectivity and reduce toxicity by taking advantage of a double-filter mechanism wherein both binding and proper positioning of the metal center are necessary for irreversible RNA cleavage activity.¹³ Therefore, nonspecific binding should not lead to side effects and toxicity. This unique mechanism of action complements current therapies and will help to combat the development of resistance.

The human La protein belongs to a class of proteins that contain RNA recognition motifs (RRMs) that interact with a variety of RNA's, including viral RNA's as well as RNA polymerase III transcripts.¹⁴ It is also necessary for HCV IRES mediated translation.¹⁵ LaR2C is a peptide derived from the RRM2 of La protein and is responsible for most of the binding to SLIV.¹⁶ Further truncation of the LaR2C provides a 7-mer that retains most of the binding affinity.¹⁷ Cryoelectron microscopy (cryo-EM) revealed the structure of the IRES bound to the 40S ribosomal subunit, but at low resolution.¹⁸ Recent crystallographic studies of the HCV IRES pseudoknot domain has provided a superimposition of the structures of the individual domains providing further structural insight into the cryo-EM data.¹⁹ The design strategy described herein consists of the incorporation of a metal-binding ATCUN motif into the LaR2C peptide (**2**) as well as the truncated 7-mer (**3**), as shown in Figure 2.

MATERIALS AND METHODS

Materials

RNA was purchased from Dharmacon, part of Thermo Fisher Scientific (Lafayette, CO). Peptides were purchased from Genemed Synthesis Inc. (South San Francisco, CA). The sequence used for the IRES SLIIB RNA was 5'-fluorescein-GGCAGAAAGCGUCUAGCCAUGGCGUUAGUAUGC C-3', for the IRES SLIV RNA was 5'-fluorescein-GGACCGUGCACCAUGAGCACGAAUCC-3', and for the HIV RRE was 5'-fluorescein-UUGGUCUGGGCGCAGCGCAAGCUGACGGUACAGGCC-3'. The sequences used for calibration of the MALDI-MS instruments included: (GU)₃, (GU)₉, (GU)₁₄, and (GU)₂₀. All RNA for binding and cleavage reactions was annealed by heating to 95 °C and then cooled slowly to room temperature before use. Sodium chloride, sodium hydroxide, and acetonitrile were purchased from Fisher, while HEPES, ammonium citrate, and 3-hydroxytyrosine were purchased from Sigma. C₁₈ ZipTips were obtained from Millipore. All experiments were performed using diethyl pyrocarbonate (DEPC) treated water and autoclaved pipette tips and tubes.

pUC19 Isolation

Plasmid pUC19 DNA was isolated from a transformed DH5α *Escherichia coli* cell line and purified by use of a Qiagen maxiprep kit. The plasmid was eluted with 20 mM HEPES and 100 mM NaCl (pH 7.4) instead of TE-Tris-EDTA buffer. The isolated DNA was quantified for purity and concentration by UV-Vis spectrophotometry.

Copper Peptides

Peptide concentrations were calibrated by titrating a stock solution of copper (II) chloride to a fixed amount of peptide at 37 °C at 5 min intervals, and then fitting the absorbance profiles at 240 nm and 525 nm. High affinity binding yielded a sharp transition at the concentration corresponding to peptide in solution. Copper complexes of the peptides were observed to form quickly and were prepared by mixing peptide and Cu (II) in a 1.1: 1 ratio, respectively, and left to equilibrate for at least 5 min prior to use.

Binding via Fluorescence

Binding data for GGHKYKETDLLILFKDDYFAKKNEERK-amide (**2**) and GGHKYKETDL-amide (**3**) were obtained by adding serial aliquots of IRES SLIV RNA to 500 nM of the peptide and monitoring tyrosine emission ($\lambda_{\text{ex}} = 280$ nm, $\lambda_{\text{em}} = 313$ nm). Data was then fit to a one-site binding model using Origin 7.0 software.

Reaction Kinetics via Fluorescence

HCV IRES RNA cleavage was assessed *in vitro* by monitoring the change in fluorescence that accompanies reaction chemistry, using 5' fluorescein end-labeled RNA with excitation and emission wavelengths of 485 nm and 518 nm, respectively. Reactions were carried out at 25 °C in reaction volumes of 100 μL, and in the presence of 1 mM ascorbate and 1 mM H₂O₂ in HEPES buffer (pH = 7.4, 100 mM NaCl) with 1 μM fluorescein-labeled IRES SLIV. The resulting time-dependent data was analyzed according to the change in

fluorescence emission observed as the reaction occurred. Both a time-dependence and a concentration-dependence of catalyst were observed. Reactions involving one phasic were fit to a first order exponential. All fits were performed using Origin software. The initial velocity values recorded in the text and tables are an average of at least three trials.

Kinetic Reactions via PAGE Sequencing Gel

Reactions were carried out in a solution volume of 10 μL that contained 10 μM fluorescein-labeled IRES SLIV, 1 mM ascorbate and 1 mM H_2O_2 in Hepes buffer with or without 10 μM 1-Cu or 2-Cu present. The reactions were staggered with time points of 10, 20, 30, 40, 50, 60, 90, 120 min. Samples were then incubated with 5 μL of 47.5% formamide, 0.5 mM EDTA and 20 % glycerol solution and heated at 65 $^\circ\text{C}$ to denature the samples before loading 8 μL onto a 20% PAGE gel. The gel was imaged on a GE Typhoon scanner. The gel was quantified by use of Quantity One (Biorad) to determine the relative concentration of the full-length. The values were then plotted to determine the initial rates which were performed in Origin software using either an exponential decay or linear fit.

pUC19 Kinetic Reactions via Gel

Reactions were carried out in a solution volume of 10 μL that contained 10 μM base pair concentration of pUC19 and 10 μM of complex in the presence of 1 mM ascorbate and 1 mM hydrogen peroxide. Reactions were staggered with time points of 10, 20, 30, 40, 50, 60, 75, 90 and 120 min. A 5 μL volume of a solution of 47.5% formamide, 0.5 mM EDTA and 20 % glycerol solution was then added, and from this mixture, 7 μL of the samples were loaded onto 1% agarose gels made with gel red (2 μL /50 mL of gel solution) with Tris-EDTA-Borate buffer and run at 100 V, and imaged by use of a BioRad Gel Doc before quantification by use of Quantity One software. The intensity of the supercoiled band was multiplied by a correction factor of 1.4 to adjust for difference in Gel Red binding. {Lloyd, 1979 #24} All fits were performed using Origin software.

HIV-RRE (Stem loop IIb) Kinetic Reactions by Gel

Reactions were carried out as described above but with 10 μM FI-RRE. Samples were then incubated with 5 μL of 47.5% formamide, 0.5 mM EDTA and 20 % glycerol solution and heated at 65 $^\circ\text{C}$ to denature the samples before loading onto a 4% agarose gel. The gels were run in parallel at 100 V for 20 min and imaged on a Biorad Gel Doc before quantifying band intensities by use of Quantity One. All fits were performed using Origin software.

Mass Spectrometry

Reactions for MALDI-TOF analysis were performed as described earlier³ by use of 10 μM fluorescein-labeled IRES SLIIb with 10 μM copper-peptide, and incubated for up to 90 min. Reactions were then quenched by placing on ice and desalted by use of C_{18} ZipTips from Millipore Co. prior to mass spectrometric analysis. ZipTips were wetted with a 50:50 mixture of acetonitrile:water and equilibrated with 2 M triethylammonium acetate (TEAA), pH 7.0. The reaction mixture was then bound to the ZipTip, washed with nanopure water, and eluted with 50:50 acetonitrile:water. Samples were spotted onto a Bruker ground steel 96 target microScout plate by first spotting with 1 μL of the 0.3 M 3-hydroxypicolinic acid

(HPA), 30 mM ammonium citrate matrix solution in 30% acetonitrile, drying, then spotting with 1 μ L of a 2:1 RNA:matrix mixture, and allowed to dry. A calibration mixture containing 4 RNAs covering a range of molecular weights, namely (GU)₃, (GU)₉, (GU)₁₄, (GU)₂₀, with molecular weights of 1,892.1, 5,800.4, 9,057.3, 12,965.6 amu, respectively, was used to calibrate the instrument. All MALDI-TOF MS analysis was performed on a Bruker MicroFlex LRF instrument, equipped with a gridless reflectron, using negative ion mode and reflectron mode. The pulsed ion extraction time was 1200 ns. Data analysis was performed using Bruker flexAnalysis software. Only m/z values > 1500 amu were considered, since excessive spectral crowding occurred at lower m/z ranges. Reaction peaks were compared to controls containing RNA alone and RNA in the presence of 1 mM ascorbate, 1 mM H₂O₂ and new peaks were identified as products of cleavage by the metalloprotein which showed time-dependence.

HCV Cellular Replicon Assay

A stable cell line ET (luc-ubi-neo/ET), a Huh7 human hepatoma cell line that contains an HCV RNA replicon with a stable luciferase (Luc) reporter and three cell culture-adaptive mutations was employed in the assay. The HCV RNA replicon antiviral evaluation assay examined the effects of compounds at six half-log concentrations each. Human interferon alpha-2b was included in each run as a positive control compound. Sub-confluent cultures of the ET line were plated out into 96-well plates that were dedicated for the analysis of cell numbers (cytotoxicity) or antiviral activity, and various concentrations of metallodrugs and controls were added to the appropriate wells the following day. Cells were processed 72 h later when the cells were still sub-confluent. Six half-log serial dilutions of the compound were performed, and values derived for IC₅₀ (the concentration that inhibited virus replication by 50%), TC₅₀ (the concentration that lowered cell viability by 50%) and TI (the selectivity index: TC₅₀/IC₅₀). HCV RNA replicon levels were assessed as the replicon-derived Luc activity. The toxic concentration of drug that reduced cell numbers (cytotoxicity) was assessed by use of the CytoTox-1 cell proliferation colorimetric assay (Promega).

RESULTS and DISCUSSION

Binding and reactivity toward IRES SLIV

Dissociation constants for binding of **2** and **3** to SLIV RNA were obtained by serial addition of RNA to peptide and following tyrosine emission as previously described for binding of **1** to SLIIb RNA.³ A sample plot is shown in Figure SM1 and the data are summarized in Table 1. Both **2** and **3** displayed similar K_D values for binding to SLIV RNA.

Binding of LaR2C to SLIV is already well established,¹⁶ and is confirmed by the *in vitro* fluorescence assays described in Table 1 for both peptides **2** and **3**. However, the similarity in affinity further suggests a binding model focused on the KYKETDL motif at the N-terminus of the LaR2C peptide. The NMR structure of the human La protein (PDB 1S79) is available¹⁵ and is illustrated in Figure 3, with the region corresponding to the LaR2C peptide highlighted in green and purple. The region corresponding to the 7-mer KYKETDL-amide that appears to dominate binding to the SLIV RNA is shown in green. Copper binding

appears to enhance affinity, presumably through copper coordination, with an average K_D for **3**-Cu, for example, of $1.2 \pm 0.2 \mu\text{M}$ (Figure SM17).

To determine the ability of these compounds to catalytically degrade RNA, cleavage activity was determined by use of an *in vitro* fluorescence assay with 5'-fluorescein-labeled SLIV RNA, as reported for Cu-GGHYrFK-amide.^{2, 3} Time-dependence plots were found to display first order exponential behavior. These initial rates were then plotted as a function of catalyst concentration (Figure 5) and the pseudo Michaelis-Menten parameters are summarized in Table 2. It is noteworthy that the k_{cat} 's are similar for **2**-Cu and **3**-Cu, suggesting similar positioning relative to scissile bonds on the RNA. However as result of the smaller targeting domain for **3**-Cu compared to **2**-Cu, there is a decrease in the binding affinity as reflected in the K_M , which is most notable in the catalytic efficiency term (k_{cat}/K_M).

To further investigate the binding and mechanism of reaction, the salt dependence of the reaction rate constant was determined for **3**-Cu with SLIV RNA (Figure 6) and showed a significant dependence on [NaCl], most likely reflecting interference with electrostatic interactions to the negatively-charged RNA substrate at higher salt concentration.

Kinetics of **2**-Cu and **3**-Cu via PAGE

SLIV RNA cleavage was also examined for both **2**-Cu and **3**-Cu through sequence PAGE gels (Table 3 and Figure SM3). Inspection shows that both complexes accelerate cleavage of the SLIV IRES RNA with similar rates of $349 \pm 93 \text{ nM/min}$ and $314 \pm 76 \text{ nM/min}$, respectively, relative to $7 \pm 6 \text{ nM/min}$ observed for the control with co-reagents only. These results are consistent with the initial rates exhibited in the fluorescence assays (Table 3). Closer examination of the gel reveals product bands that are faintly observed, but are not sufficiently resolved for accurate quantification.

Selectivity of **2**-Cu and **3**-Cu

To probe the selectivity of **2**-Cu and **3**-Cu for SLIV RNA, additional oligonucleotides, including the rev-response element (RRE) RNA of HIV stem-loop II, and pUC19 (supercoiled DNA) were each tested (Table 4 and Figure SM4 and SM5). No significant cleavage was observed with Fl-HIV RRE and very limited disappearance of supercoiled pUC19 with time. In both cases the rate of cleavage was found to be less than 20 nM/min , relative to the enhanced rate of $300\text{--}500 \text{ nM/min}$ for HCV SLIV. Additional data that addresses potential problems with non-selective cleavage are addressed in the section on cellular replicon assays.

These observations reflect an important and unique feature of the approach, namely, the double-filter mechanism. While either of these compounds could bind to other non-specific targets and act as reversible inhibitors, nevertheless, under sub-saturating conditions, it is only when these complexes cleave the RNA or oligonucleotide target that they become the irreversible inhibitors that is the unique property of the catalytic metallodrug design. For this scenario to occur, not only do the metallodrug complexes need to bind, but they must also

place the catalytic metal in an orientation to produce/provide effective cleavage chemistry on the target (Figure SM 2).

Mass Spectrometric Analysis of RNA Cleavage

To further probe the mechanism of reaction and map potential cleavage sites, MALDI-TOF mass spectrometry was performed for the reaction of **2**-Cu and **3**-Cu with SLIV. MALDI-TOF analysis of RNA cleavage products is complicated by the presence of a large number of fragmentation peaks that are present even in the absence of catalyst and occur as a result of ionization from the instrument as we have previously described.²⁰ To circumvent the problem of identifying actual metalloprotein-mediated cleavage products, only those peaks that were new (not contained either in controls of RNA alone or RNA in the presence of the coreagents ascorbate and H₂O₂ in the absence of catalyst) and displayed a time-dependent change in intensity were considered. Illustrative time-dependent mass spectra are shown in Figure 9 and peak assignments for each time point are detailed in Tables SM1 and SM2.

Most of the observed products occur within a common locus (Figure 9). For reactions with **2**-Cu, the most significant sites of reactions are at G15 and A16, which represent the last nucleotide, and the nucleotide immediately following, the internal AUG start codon sequence (from site 13 to 15). For **3**-Cu the main cleavage occurs at G8 which is part of one of the GCAC motifs. For both of these complexes, reactivity is centered on the GCAC domains that are known to be important for ribosome assembly (Figure 8 and 9). A visual representation of the initial rates is available in Figure 9 in both a 2D and 3D representation, with the actual values summarized in Table 5. It is important to keep in mind that the proposed 3D structure is not based on crystallographic or NMR information, but rather was derived from linear least squares fitting of SAXS data information, and is an average of the relative populations of various structures.

The data appears to suggest that each complex reacts at sites flanking the two GCAC sites ; primarily at the top of the stem-loop for **2**-Cu (with modest activity in the lower domain), and in the lower domain for **3**-Cu. The 3D structural model, based on SAXS studies (Figure 9), shows the SLIV domain to be already bent in a manner that would allow access for the catalytic center to the domains flanking the GCAC sites. Most likely the C-terminal amino acids in the longer **2**-Cu metalloprotein provide additional contacts that adjust the orientation and promote more efficient chemistry for **2**-Cu, as reflected by comparison of both the k_{cat}/K_M ratios (Table 2) and relative initial rates from MALDI experiments (Figure 9 and Table 5).

Additional mechanistic insight was obtained by examining the types of overhang produced by each reaction (Figure 10). Because little work has been reported for direct characterization of the kind of overhang expected from RNA cleavage reactions, product patterns were based on those reported for DNA fragmentation.²² Of interest is the rate at which different overhangs are produced. In general, the relative rates are similar to both complexes across the different overhangs observed (Table 6). This is not surprising as their reactivity has been approximately the same as determined by other techniques. This also includes the general sphere of reactivity towards SLIV. **2**-Cu appears to be slightly faster in the production of 2',3'-cyclic phosphates and 3'-phosphates, which suggests that additional

contacts from the longer peptide stabilizes the metallopeptide-RNA complex to perform slightly more efficient chemistry. The proposed mechanism of interaction of action of these compounds have been reported and is available in Figure SM 15.³

Cellular Replicon Activity

After binding and reactivity were established *in vitro*, the activity of **2**-Cu and **3**-Cu were evaluated in cell culture by use of a cellular HCV replicon assay that mimics the native HCV replication process and is accepted by the FDA as a measure of the efficacy of potential HCV treatments. Results are shown in Table 7 and are compared to the data previously reported for **1**-Cu.² Both metallopeptides showed activity similar to **1**-Cu and, as for **1**-Cu, no activity was observed in controls of the RNA binding domain alone, Cu-GGH alone, free copper, or the peptide without copper. Recombinant IFN α -2b was used as a positive control.

The absence of replicon activity for the metal-free peptides, the GGH metal binding domain, and RNA binding domains alone are each consistent with **2**-Cu and **3**-Cu reacting in the cellular assays by targeted catalytic cleavage, as observed *in vitro*, as opposed to nonspecific reaction or competitive inhibition. The absence of replicon activity by **2** (without copper) contrasts with a previous report of cellular translational inhibition by the LaR2C peptide.¹² Given that the HCV cellular replicon assays used herein are the standard assays accepted by the FDA, while the earlier studies were performed using a simpler, less robust assay, these differences can reasonably be attributed to the use of distinct cellular assays. This cellular replicon activity, along with the lack of toxicity, further supports a model where these metallopeptides exhibit specificity for HCV RNA.

The metallopeptides described herein expand the available pool of potential catalytic metallodrugs for the treatment of HCV infection. The results help to validate the targeting of IRES RNA by **2**-Cu and **3**-Cu as a possible therapeutic approach to the treatment of HCV infection and further demonstrate the general applicability of the catalytic metallodrug approach to drug design. Building on the prior report of SLIIB RNA cleavage by Cu-GGHYrFK-amide (**1**-Cu), and the demonstration of its effectiveness as an antiviral agent when used in combination with rIFN α -2b, the current results suggest that **2**-Cu and **3**-Cu could also find application in combination with current therapeutics, which now include both protease and polymerase inhibitors. In particular, their novel mechanism of action renders them ideal for use in combination treatments. Alternatively, such compounds could be used in tandem with compounds such as **1**-Cu as a mixture that targets various IRES stem loop structures.

Conclusions

Several currently approved FDA drugs have developed resistance to HCV and, therefore, the need for a multiple target approach has only grown. To circumvent resistance problems, HCV treatment will require multidrug therapy. Reported here are two complexes based upon the RRM2 of human La protein which show selectivity towards stem-loop IV of the HCV IRES. Binding affinities without the copper are $\sim 10 \mu\text{M}$. Catalytic parameters for the copper complexes yielded $k_{\text{cat}} \sim 1 \text{ min}^{-1}$, and $k_{\text{cat}}/K_{\text{M}}$ of $0.21 \mu\text{M}^{-1} \text{ min}^{-1}$ and $0.07 \mu\text{M}^{-1} \text{ min}^{-1}$ for

2-Cu and **3-Cu**, respectfully. An FDA accepted cellular replicon assay determined the antiviral activity to be 0.75 μM and 2.17 μM for **2-Cu** and **3-Cu** respectfully, while showing no toxicity to cells up to the maximum concentration of 50 μM .

Drugs that target SLIV of the HCV IRES RNA represent a novel approach and mode of action, as well as a distinct therapeutic target for the treatment of HCV infection and have the potential to complement both current treatments and those still in development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants from the National Institutes of Health [HL093446 and AA016712]. The Bruker Microflex instrument used for all MALDI-TOF analysis was provided by a grant from the Ohio BioProducts Innovation Center. We would like to thank Jeff Joyner for his development of MassDaddy for MALDI-TOF fragmentation assignments. We thank Dr. Zhuhui Huang and staff at Southern Research Institute for performing the cellular replicon assays. We thank Jessica Alexander for the purification and isolation of pUC19 and Insiya Fidai for the CD experiments.

References

1. Shimakami T, Lanford RE, Lemon SM. *Curr Opin Pharmacol*. 2009; 9:537–544. [PubMed: 19762279]
2. Bradford S, Cowan JA. *Chem Commun*. 2012; 48:3118–3120.
3. Bradford SS, Ross MJ, Fidai I, Cowan JA. *ChemMedChem*. 2014; 9:1275–1285. [PubMed: 24756921]
4. Gokhale N, Cowan J. *Chem Commun*. 2005:5916–5918.
5. Joyner JC, Cowan JA. *J Am Chem Soc*. 2011; 133:9912–9922. [PubMed: 21585196]
6. Joyner JC, Hocharoen L, Cowan JA. *J Am Chem Soc*. 2011
7. Suh J, Chei WS. *Curr Op Chem Biol*. 2008; 12:207–213.
8. Suh J, Chei WS, Lee TY, Kim MG, Yoo SH, Jeong K, Ahn JY. *J Biol Inorg Chem*. 2008; 13:693–701. [PubMed: 18320241]
9. Suh J, Yoo SH, Kim G, Chae PS, Lee TY, Lee J, Lee J, Jang YA, Ko EH. *Angew Chem*. 2007; 119:7194–7197.
10. Sarnow P. *Journal of Virology*. 2003; 77:2801–2806. [PubMed: 12584303]
11. Subramanian N, Mani P, Roy S, Gnanasundram SV, Sarkar DP, Das S. *J Gen Virol*. 2009; 90:1812–1819. [PubMed: 19297606]
12. Pudi R, Ramamurthy SS, Das S. *J Gen Virol*. 2005; 79:9842–9853.
13. Hocharoen L, Cowan JA. *Chem Eur J*. 2009; 15:8670–8676. [PubMed: 19685535]
14. Qin N, Chen Z, Yao HP, Yang ZG, Liu KZ, Wu LL. *World J Gastroenterol*. 2004; 10:2050–2054. [PubMed: 15237432]
15. Alfano C, Sanfelice D, Babon J, Kelly G, Jacks A, Curry S, Conte MR. *Nat Struct Mol Biol*. 2004; 11:323–329. [PubMed: 15004549]
16. Pudi R, Abhiman S, Srinivasan N, Das S. *J Biol Chem*. 2003; 278:12231–12240. [PubMed: 12540850]
17. Mondal T, Ray U, Manna AK, Gupta R, Roy S, Das S. *Journal of Virology*. 2008; 82:11927–11938. [PubMed: 18829760]
18. Siridechadilok B, Fraser CS, Hall RJ, Doudna JA, Nogales E. *Science*. 2005; 310:1513–1515. [PubMed: 16322461]

19. Berry KE, Waghay S, Mortimer SA, Bai Y, Doudna JA. *Structure*. 2011; 19:1456–1466. [PubMed: 22000514]
20. Andersen TE, Kirpekar F, Haselmann KF. *J Am Soc Mass Spectr*. 2006; 17:1353–1368.
21. Perard J, Leyrat C, Baudin F, Drouet E, Jamin M. *Nature Commun*. 2013; 4:1612. [PubMed: 23511476]
22. Pogożelski W, Tullius T. *Chem Rev*. 1998; 98:1089–1107. [PubMed: 11848926]
23. Joyner JC, Keuper KD, Cowan JA. *Nucl Acids Res*. 2013; 41:e2. [PubMed: 22941655]
24. Joyner JC, Keuper KD, Cowan JA. *Chem Sci*. 2013; 4:1707–1718. [PubMed: 23626900]

Figure 1.

The highly structured Internal Ribosome Entry Site (IRES) in the 5' untranslated region of hepatitis C viral mRNA (left). The secondary structure (center), and three dimensional structure based on SAXS, small angle x-ray scattering, data²¹ (right) of stem-loop IV (SLIV) which contains the internal start codon (AUG), and residues 5'-G₈ to C₁₁-3' and 5'-G₁₇ to C₂₀-3' that correspond to the known binding site on the human La protein.

Figure 2.

Structure of the metal binding ATCUN motif with targeting R groups that define the domains that recognize the GCAC sequences of SLIV. Complex **1-Cu** (containing a D-configuration arginine)² binds selectively to SLIIb IRES RNA, while **2-Cu** and **3-Cu** are based on the LaR2C peptide that recognizes HCV IRES SLIV.^{16,17}

Figure 3.

NMR solution structure of the human La protein showing all 20 structures including the secondary elements superimposed (based on PDB 1S79) with the portion corresponding to LaR2C, **2**, highlighted (green and purple). The green domain corresponds to the truncated 7-mer, **3**. The left side shows the portion of the La protein from which the compounds were derived, as well as the highly conserved structure (secondary structure, top and atomic resolution, bottom).

Figure 4.
Sample kinetic trace for the reaction of 7.5 μM 2-Cu with HCV IRES SLIV showing monophasic behavior with the fit shown in red.

Figure 5.
Pseudo Michaelis-Menten plots for reaction of **2-Cu** (left) and **3-Cu** (right) with HCV IRES SLIV.

Figure 6. Variation of initial rate with [NaCl] for reaction of **3**-Cu with HCV IRES SLIV. Conditions for assay were 20 μM **3**-Cu, 1 μM fluorescein labelled SLIV, 1 mM ascorbate and 1 mM hydrogen peroxide in HEPES buffer pH 7.4.

Figure 7.

Time-dependence of MALDI-TOF chromatograms following reactions catalyzed by **2**-Cu (top) and **3**-Cu (bottom) with SLIV RNA in the presence of coreagents. Time points from front to back are 2, 10, 20, 30, 45, 60, and 90 min. The y-axis is the normalized fraction of the total intensity at each time point and does not account for mass bias. The initial rate was determined at each position, and those that showed a measurable time-dependence are summarized in Table 5. The contributions of 3' and 5'-overhangs are available in Figures SM 6 and SM 7.

Figure 8.

Three and Two dimensional representations of SLIV RNA. The three dimensional representation is based on a SAXS structure predicted by Perard *et al.*²¹ The two dimensional representation is based on VARNA structure prediction. The 5'-GCAC-3' sequences highlighted correspond to potential target recognition sites for **2**-Cu and **3**-Cu (blue and orange), while, the AUG start codon (green) at the top of the hair-pin is also highlighted.

Figure 9.

Mass spectrometric analysis of cleavage products for reactions of HCV IRES SLIV mediated by **2**-Cu (top) and **3**-Cu (bottom), including heat maps of reactivity obtained by assessing the relative initial rate values at each nucleotide position. The black bases or gray highlighted region represents the two known binding sequences of 5'-GCAC-3' which are labelled. It can be seen that reactivity occurs on either side of this region. This suggests that the copper complexes induce a structural change which brings the two domains close in proximity which facilitates the range of cleavage. Relative Scale: Red $x > 10$, Orange $8 < x$

< 10 , Yellow $6 < x < 8$, Green $4 < x < 6$, Blue $2 < 0 < 4$, Purple $x < 2$. Values are from the initial rates observed from MALDI-MS multiplied by 1000.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 10.

Relative amount of each class of product, following catalytic reaction with **2**-Cu (top) and **3**-Cu (bottom), as observed by mass spectrometry and displayed as a function of time. Kinetic data fits are available in the supplementary material (Figure SM10 and SM11).

Table 1

Dissociation constants to HCV IRES SLIV for peptides based on LaR2C.

Peptide	SLIV K_D (μM)
[GGHKYKETDLLILFKDDYFAKKNEERK-amide] (2)	9.9 ± 0.5
[GGHKYKETDL-amide] (3)	9.7 ± 0.1

Table 2

Michaelis-Menten parameters for degradation of SLIV RNA.

Compound	k_{cat} (min^{-1})	K_{M} (μM)	$k_{\text{cat}}/K_{\text{M}}$ ($\mu\text{M}^{-1} \text{min}^{-1}$)
2-Cu	0.98 ± 0.07	4.6 ± 1.1	0.21
3-Cu	1.13 ± 0.06	17.2 ± 2.2	0.07

Table 3

Comparison of initial rate measurements obtained from fluorescence and PAGE profiles

Condition	Initial Rate (nM/min)	
	Fluorescence ^a (nM/min)	PAGE ^b (nM/min)
2-Cu	509 ± 122	349 ± 93
3-Cu	423 ± 105	314 ± 77
coreagents ^c	8 ± 5	7 ± 6

The graphs are available in supplementary material (Figures SM3, SM4 and SM5).

^aReaction conditions: 1 µM FI-HCV SLIV in the presence of 10 µM complex and 1 mM co-reagents.

^bReaction conditions: 10 µM FI-HCV SLIV in the presence of 10 µM complex and 1 mM co-reagents.

^cCoreagents added in the absence of 2-Cu and 3-Cu.

Table 4

Selectivity of **2**-Cu and **3**-Cu as represented by initial rates as monitored from gels.

Condition	Initial Rate (nM/min)		
	FI-HCV SLIV ^a	pUC19 ^b	FI-HIV RRE ^a
2 -Cu	349 ± 93	19 ± 4	7 ± 4
3 -Cu	314 ± 77	10 ± 2	14 ± 16
coreagents ^c	7 ± 6	2 ± 1	0 ± 12

The gels and the graphs are available in supplementary material.

^aReaction conditions: 10 µM of RNA or 10 µM bp of pUC19 in the presence of 10 µM complex and 1 mM co-reagents.

^bReaction conditions: 10 µM bp of pUC19 in the presence of 10 µM complex and 1 mM co-reagents.

^cCoreagents added in the absence of **2**-Cu and **3**-Cu.

Table 5

Relative initial rate for SLIV cleavage reactions mediated by **2**-Cu and **3**-Cu determined by MALDI-TOF experiments.

	Initial Rate (Normalized Intensity/min) $\times 1000$	
	2-Cu	3-Cu
G₂	0.3 ± 0.1	
A₃		9.3 ± 1.0
C₄	4.5 ± 0.1	3.3 ± 0.4
G₆	3.1 ± 0.5	
G₈		37.1 ± 8.7
A₁₀	3.5 ± 0.4	
C₁₂	6.1 ± 0.3	
G₁₅	9.1 ± 0.8	0.6 ± 0.4
A₁₆	13.8 ± 4.2	1.1 ± 0.3
G₁₇	1.4 ± 0.3	
A₁₉		0.8 ± 0.1
C₂₀		1.0 ± 0.3
G₂₁	3.1 ± 0.1	0.9 ± 0.3
U₂₄	0.5 ± 0.1	

Kinetic fits for the initial rates are available in the supplemental material (Figure SM12 for **2**-Cu and SM13 for **3**-Cu).

Table 6

Initial rates for select overhang products observed following reactions promoted by **2**-Cu and **3**-Cu as monitored by time-dependent MALDI-TOF mass spectrometry (see also Tables SM1 and SM2 and Figures SM10 and SM11).

Overhang	mass (amu)	2-Cu (%/min)	3-Cu (%/min)	Possible Mechanism
2',3'-Cyclic Phosphates	61.96	10.5 ± 4.3	4.3 ± 0.5	Hydrolysis
3'-Phosphates	79.98	6.4 ± 1.3	2.8 ± 0.2	Hydrolysis, 1', 2', 3', 4', or 5'-H abstraction ^a
3'-Phosphoglycolates	138.02	4.5 ± 1.4	4.0 ± 1.8	4'H abstraction
5-Hydroxyl	0.00	2.3 ± 0.1	1.1 ± 0.1	Hydrolysis

Overhang	mass (amu)	2-Cu (%/min)	3-Cu (%/min)	Possible Mechanism
79.98	2.8 ± 2.5	6.1 ± 1.4	Hydrolysis, 1', 2', 3', 4', or 5'-H abstraction ^a
5'-phosphates				

^a Although 1', 2', 3', 4', or 5'-H-abstraction are possible 4'-H and 5'-H abstraction are expected to be more common as a result of greater solvent exposure.

Table 7HCV cellular replicon activity for **2**-Cu and **3**-Cu.

Compound	Antiviral IC ₅₀ (μM)	Cytotoxicity TC ₅₀ (μM)	Selectivity Index TI
[GGHKYKETDLLILFKDDYFAKKNEERK-amide]-Cu, 2 -Cu	0.75	> 50	> 67
[GGHKYKETDL-amide]-Cu, 3 -Cu	2.17	> 50	> 23
[GGHYrFK-amide]-Cu, 1 -Cu	0.58	> 100	> 172
[KYKETDLLILFKDDYFAKKNEERK-amide] (2)	> 100	> 100	na ^a
[GGH]-Cu ²⁺	> 100	> 100	na ^a
Cu ²⁺ (aq)	> 100	> 100	na ^a

^a na- not applicable